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The Evolution of Sex Determination and the DMRT1 Gene in the Japanese Gecko (Gekko japonicus)

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THE EVOLUTION OF SEX DETERMINATION AND THE *DMRT1* GENE IN THE JAPANESE GECKO (*GEKKO JAPONICUS*)

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by

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ABSTRACT

THE EVOLUTION OF SEX DETERMINATION AND

THE *DMRT1* GENE IN THE JAPANESE GECKO

(*GEKKO JAPONICUS*)

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There are different sex-determining mechanisms in our environment. They are separated into two groups known as genetic sex determination (GSD) and environmental sex determination (ESD). The most well-known mechanism in the ESD group is temperature sex determination (TSD). In this study, the presence of the Doublesex and mab-3-related Transcription Factor (*Dmrt1*) gene was observed during embryonic development, in geckos, with a TSD mechanism. To do this, I observed the rate of transcription of the *Dmrt1* gene in the Gecko species *Gekko japonicus*. Pregnant geckos were caught around Nanjing, China. Once the females laid their eggs, the eggs were then randomly placed in one of three different temperature regimes (24°C, 28°C, 32°C). The embryos, the main target, were dissected at two points during development and total RNA was extracted. Findings suggest *G. japonicus* may not be utilizing a TSD system but rather a GSD system with a TSD override.

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CHAPTER I

INTRODUCTION

Throughout the animal kingdom, it is common to find species with more than one sex. Asexual reproduction is a fairly primordial reproduction process due to the inability of the organism to evolve and vary its genes (Lloyd 1980). However, there are multiple sex-determining systems used to distinguish males from females. The most familiar system is the XY chromosome system, which is what determines the sex of humans (*Homo sapiens*). This system uses sex chromosomes, distinguished as X or Y. When two X chromosomes are present we get genotypic females and when an X and Y are present we have genotypic males. The opposite is true for a ZW chromosome system. Here we expect the homogametic ZZ to produce males, and the heterogametic ZW to produce females. These chromosome systems are known as genotypic sex determination (GSD). The XY mode of sex determination can be found in all monotremes, marsupials and placental mammals. The ZW system is found in all birds, some reptiles and fish (Rice 1984; Janzen & Krenz 2004).

The least known sex-determining mechanism is environmental sex determination (ESD), which is when abiotic factors in an organism's environment, namely temperature, influence the sex of the organism. Temperature sex determination (TSD) can skew the typical 1:1 (male-female, MF) sex ratio observed in GSD. There are three different types of skews typically observed: FM, FMF and MF. FM occurs when low temperatures yield a female bias and high temperatures yield a male bias. When both high and low temperatures produce a female bias and intermediate temperatures a male bias, it is

known as FMF. MF displays a male bias at lower temperatures and a female bias at higher temperatures (Ewert *et al*. 1994). TSD is displayed in some turtles, lizards, fish and all crocodiles (Bull 1980; Crews *et al*. 1994; Janzen & Phillips 2006; Trukhina *et al*. 2013). The reptilian family *Gekkonidae* displays both GSD (XY and ZW systems) and TSD mechanisms (Sarre 2011). Although there are two distinct types of sex determination, it is not clear whether GSD and TSD are on two opposite ends of a spectrum or if they are rather a continuum of sex-determining mechanisms (Valenzuela *et al*. 2003; Sarre *et al*. 2004).

Embryonic Development

During embryonic development in TSD organisms there is a time period in which the gonads start developing into either ovaries or testes, depending on the temperature of incubation. Once this period is over their gonadal fate is set. This time period is known as the thermo-sensitive period, or TSP. This window tends to occur during the middle third of development (Crews 1996; Janzen & Phillips 2006; Shoemaker *et al*. 2007a; Huang & Crews 2012). During the TSP period, if embryos start at one temperature extreme and are then shifted into another near the end of the TSP window, the gonads start developing into the opposite sex than they were initially developing into (Crews *et al*. 1994). What is more interesting is that GSD individuals, such as the newt *Pleurodeles waltl,* also have a TSP with a ZW chromosome system. Temperature can override the sex chromosomes so that genotypic ZW females are all transformed into phenotypic males (Kuntz *et al*. 2003). GSD override has also been observed in the skink *Bassiana duperreyi,* and the lizard *Pogona vitticeps* (Shine *et al.* 2002; Quinn *et al*. 2007). The reason that many of these

switches in sex can occur during early embryonic development is the presence of a bipotential gonad.

Within the first third of development embryos have a bipotential gonad also known as the bipotential primordium genital ridge. This bipotential gonad has the ability to develop into either of two mutually exclusive directions, which we know as the ovary and testes. At this point in development embryos have the potential to become either male or female, whether sex chromosomes are present or not. Once the embryos enter the TSP the bipotential gonad starts to develop sertoli cells and morph into testes, or they develop granulosa cells and morph into ovaries, and the sex of the organism is determined (Shoemaker et al., 2007a; Rhen & Schroeder, 2009).

Sex-Determining Genes

As discussed earlier, there are two different types of GSD mechanisms. Many people are familiar with mammalian sex chromosomes, which have an XY system. This is when males are heterogametic (XY), and females are homogametic (XX). Whereas in other organisms, like avian species and some reptiles, we find that there is a ZW sex chromosome system. This is when females are heterogametic (ZW) and males are homogametic (ZZ). Genes on the sex chromosomes can work either by a dominant effect or a dosage effect. As a generalization in an XY system, males have a sex-determining region of the Y chromosome (*SRY)* gene that females do not have, which turns on another gene and leads to male testis development. This is an example of a dominant effect. The dosage effect occurs in a ZW system and applies only if both males and females have the same gene on their Z chromosome. Since males have two Z chromosomes, there is a

higher rate of transcription of the gene, which leads to more masculine features (Ezaz *et al*., 2009; Graves, 2013). The reason the dosage effect does not typically occur in a XY system is due to X inactivation. Each cell in a female's body inactivates one of the X chromosomes so that only one X chromosome gene is transcribed. Interestingly, in marsupials it is always the paternal X chromosome that is inactivated, but in mammals it is random (Bainbridge 2004). TSD individuals do not have sex chromosomes and therefore, use another system to determine sex. What this system might be remains a mystery.

When observing mammalian genetics with an XY system, it has been found that the *SRY* gene on the male Y chromosome is responsible for testicular development. This gene triggers the cascade of events that lead to the production of testicular tissue in males (Goodfellow & Lovell-Badge 1993; Crews *et al*. 1994). However, in TSD individuals there are other genes responsible for testis and ovary development. Many of these genes also appear in GSD individuals, but they have different upstream versus downstream roles.

Some of the most popular genes involved in sex determination in the animal kingdom are the genes Sry-like HMG-box 9 (*Sox9)* and doublesex mab-3-related transcription factor 1 (*Dmrt1)*. Both genes are supported in many species to be a male-determining gene, but the order in which they become transcriptionally activated may differ by species (Shoemaker *et al*. 2007a; Smith *et al*. 2009; Trukina *et al*. 2013). In Avian species, *Dmrt1* is similar in function to the *Sry* gene in mammals. More specifically, chickens (*Gallus gallus*) have been shown to require the *Dmrt1* gene for

male development in a ZW system (Smith *et al*. 2009). Without this gene, downstream genes will not be activated, which means male gonads would not develop. Hence the term for these genes was coined “master sex-determining” (Meise *et al*. 1998). Not only is *Dmrt1* required for avian sex determination, but it also plays a role in male gonad development in many vertebrates including mammals, reptiles and fish. This is curious because this gene is not limited to GSD individuals but also organisms that display a TSD mechanism (Raymond *et al*. 2000; Murdock & Wibbels 2006; Shoemaker *et al*. 2007a; Trukina *et al*. 2013).

Similar to *Dmrt1*, *Sox9* is displayed across the animal kingdom in both GSD and TSD individuals (Shoemaker *et al*. 2007b). When studying TSD, the red-eared slider turtle (*Trachemys scripta)* is the primary model organism (Murdock & Wibbels 2006). In 2007, Shoemaker and colleagues studied the role of *Sox9* and *Dmrt1* in *T. scripta.* Before this research was done it had previously been determined that *Sox9* and *Dmrt1* play a role in male development. This study found that *Sox9* had an upstream role in male gonad development, similar to mammals. They also found that *Dmrt1* seems to have an upstream role, whereas in mammals *Dmrt1* has a downstream role. These genes are all linked to male gonad development, but there are also female specific genes linked to female gonad development. In the past females were thought to be the “default” sex since males expressed the *SRY* gene leading to the development of masculine features (Bainbridge 2003). However, current data suggest female development may also require active signaling molecules for proper ovarian development.

Within the last 10 years the importance of the genes forkhead box L2 (*FoxL2),* wingless type MMTV integration site family, member 4 (*WNT4)* and *P450* aromatase (also known as *CYP19*) to female gonad development has been recognized. In the snapping turtle *FoxL2* is expressed 25-fold in ovaries versus testes (Rhen *et al*. 2007). This suggests that *FoxL2* is associated with ovarian development in the embryonic stage. In mice, if *FoxL2* is not activated the ovaries will fail to develop in females (Rhen & Schroeder 2010). Another gene that is upregulated in females is known as *WNT4* (Trukhina *et al*. 2013). If *WNT4* is inactivated in humans, it will reverse the sex of an XX female into an XX phenotypic male. Also, when the gene is duplicated and overexpressed, an XY male will become an XY phenotypic female (Jordan *et al*. 2001), which advocates that *WNT4* is needed for female gonad development. Additionally, the gene *CYP19* codes for an enzyme known as *P450* aromatase. When *CYP19* is knocked out in mice, males show almost normal phenotypic characteristics, whereas females are sterile due to underdeveloped sex organs (Fisher *et al*. 1998). This enzyme regulates the estrogen that is present in the gonads by converting androgens (i.e. testosterone) into estrogen.

By preventing the synthesis of testosterone into estrogen during embryonic development, female chick embryos show a reversal in sex and display male gonads once hatched. This has also been observed in TSD species. When incubation temperatures are set to a temperature with a male bias, and then estrogens are placed on the eggshells during development, a female bias will be displayed instead (Jeyasuria & Place 1998). More recently researchers are starting to believe that TSD is more reliant on the amount

of estrogen being produced. Rather than having a master sex-determining gene, the quantity of hormones is the master switch. It has been shown in many cases that the application of estrogen-like compounds to TSD eggs leads to all females with normal reproductive abilities (Lance 2009).

Not only do these genes determine the sex of an organism, but they have also been shown to regulate the expression of each other. *Dmrt1* has been found to inhibit the expression of *FoxL2* in mice. This prevents the transformation of the male Sertoli cells, found only in the testes, into female granulosa cells, found only in the ovaries (Defalco 2014; Minkina *et al*. 2014).

Hormone Treatments

Hormones play a major role in sex determination. As early as 1939, Burns used estrodiol dipropionate to reverse the sex of the American opossum (*Didelphys virginiana*). Then about 60 years later, Coveney *et al*. (2001) gave newborn Tammar Wallaby's estradiol benzoate for 25 days, and found that genotypic males shifted to become phenotypically female. Recently, the application of 17β-estradiol had been found to significantly decrease the expression of *Dmrt1* and *Sox9* in *T. scripta* (Murdock & Wibbels 2006; Barske & Capel 2010). This shows that estrogen has the potential to down regulate genes in TSD species, not to mention an increase in the enzyme activity of aromatase in female producing temperatures in *T. scripta* (Crews *et al*. 1994). This is important because of its regulation and synthesis of estrogen in the ovary.

Geckos

In the family *Gekkonidae* it is known that two species use the XY system, 11 use the ZW system and eight species are TSD out of the known 794 species in this family (Ezaz *et al*. 2009). One of the most studied geckos in this family is *Gecko hokouensis*, which has a ZZ/ZW mechanism. Through cloning and chromosome mapping it has been found that the avian ZW chromosomes are highly conserved to the ZW chromosomes found in *G. hokouensis*, suggesting this is an example of divergent evolution (Kawai 2009). While other lizards, such as the Australian dragon lizard, show no homology to other ZW chromosomes and seem to be an example of convergent evolution (Ezaz *et al*. 2009b). Due to the homology of ZW chromosomes of the chicken (with a *Dmrt1* sex-determining gene) and *G. hokouensis*, it seems likely that *Dmrt1* plays an upstream role and serves a similar purpose in this gecko species (Smith *et al*. 2009).

G. japonicus has been considered to have a TSD mechanism with a pattern of FMF, for the last 30 years (Tokunaga 1985). However, about a decade before that a study found that *G. japonicus* did indeed have sex chromosomes present (Yoshida & Itoh 1974). In 1986, Chen and colleagues performed their own karyotype and did not observe any sex chromosomes, although this remains unknown since no one knows where the karyotyped geckos were collected in the 1974 study. Even so, there are many speculations as to why there was a difference observed, such as hybridization of species in Japan and China (Gamble 2010). We already know that reptiles with GSD mechanism can be overridden by temperature, which makes it harder for us to really know whether *G. japonicus* is truly TSD or just GSD with a TSD override.

In a more recent study it was found that there were significantly more females at the high and low end of the temperature spectrum. However, the intermediate temperature displayed almost a 1:1 ratio. This is not consistent with the TSD mechanism proposed earlier by Tokunaga in 1985. In this newer study, when observing egg size, it was found that larger eggs are more likely to produce females, and when looking at the intermediate temperature the larger eggs also produced males, supporting the old FMF TSD mechanism (Ding *et al*. 2012).

For my study, I would like to observe how some sex-determining genes are involved in this TSD species. We now know how hormones and temperature affect the sex of *G. japonicus* offspring, but we have little knowledge about the gene expression at present. My goal is to determine how these genes may differ in expression under different temperature regimes. If this is indeed the case, my study will provide further evidence in support of the TSD mechanism of *G. japonicus.* Yet, due to the complexity of sex-determining mechanisms we may find that sex-determining systems cannot be so easily established.

CHAPTER II

SEX DETERMINATION PATTERNS IN THE JAPANESE GECKO (*GEKKO JAPONICUS*)

SEX DETERMINATION PATTERNS IN THE JAPANESE GECKO (*GEKKO JAPONICUS*)

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Abstract

There are different sex-determining mechanisms in our environment, which are separated into two groups known as genotypic sex determination (GSD) and environmental sex determination. The most well-known mechanism in the ESD group is temperature-dependent sex determination (TSD). In this study, the presence of the Doublesex and mab-3-related Transcription Factor (*Dmrt1*) gene was observed during embryonic development in geckos with a TSD mechanism. To do this, I observed the rate of transcription of the *Dmrt1* gene in the Gecko species *Gekko japonicus*. Pregnant geckos were caught around Nanjing, China. Once the females laid their eggs, the eggs were then randomly placed in one of three different temperature regimes (24°C, 28°C, 32°C). The embryos, the main target, were dissected at two points during development and total RNA was extracted. A relative rate of transcription of the *Dmrt1* gene was assessed using quantitative PCR (qPCR). I was not able to quantify the use of the *Dmrt1* gene between males and females. However, my study does not allow me to exclude the possibility that *G. japonicus* may use a GSD system with a TSD override, instead of the earlier proposed TSD system.

Keywords:

Sex Determination	DMRT1	Temperature-dependent sex determination
Reptile	Gekko japonicus	
RT-PCR/qPCR		

Introduction

Reptiles are unique in the animal kingdom due to the diversity in sex determination mechanisms. Some reptilian species have XX/XY chromosomes with male heterogamety, whereas other species possess ZZ/ZW chromosomes with female heterogamety. The mechanism in animals where the genes on the sex chromosomes determine the sex of an organism is known as genotypic sex determination (GSD). GSD is the observed mechanism for sex determination in all mammals including monotremes, marsupials and placental mammals. However, some organisms have a mechanism of sex determination that does not involve genes located on sex chromosomes. Environmental influences, most commonly temperature, are known to cause a shift from the normal 1:1 (male-female) sex ratio in offspring during incubation in many reptiles (Rhen & Schroeder 2010). This sex-determining mechanism is known as temperature-dependent sex determination (TSD) and is displayed in some turtles, lizards, fish and all crocodiles (Crews *et al*. 1994; Janzen & Phillips 2006, Trukhina *et al*. 2013).

Even though there are two types of sex determination, there is not a clear distinction between GSD and TSD. There seems to be more of an interaction between genes and temperature than previously thought (Valenzuela *et al*., 2003; Sarre *et al*., 2004). If genes were thought of as a railroad switch and one track of genes led to producing males and the other females, then temperature could be the trigger that flips the switch one way or the other. Not only can temperature influence the sex of an organism, it can also override genotypic expression in the skink *Bassiana duperreyi* and the lizard *Pogona vitticeps* (Shine *et al.*, 2002; Quinn *et al*., 2007). Shine and colleagues found that temperature can override sex chromosomes in 2002, and they also found that

egg size affected the sex of the skink as well. Egg size has also been known to influence the sex of a TSD gecko, *Gekko japonicus* (Ding *et al*. 2012). Hormone treatments, such as estrogens, can also override GSD as demonstrated in the lizard *Bassiana duperreyi* (Radder *et al*. 2008). These hormone treatments have been shown to sway the sex of an organism, whether it uses a GSD or TSD system (Radder *et al*. 2008; Ding *et al*. 2012).

The reptilian family Gekkonidae displays both GSD (XY and ZW systems) and TSD mechanisms (Sarre 2011). Out of the known 794 Gekkonidae species, two species use an XY sex determining system and 11 use the ZW GSD system, while eight species utilize a TSD system (Ezaz *et al*. 2009). One of the most studied geckos in this family is *Gecko hokouensis*, which has a ZZ/ZW mechanism. Through cloning and chromosome mapping it has been found that the avian ZW chromosomes are highly conserved to the ZW chromosomes found in *G. hokouensis*, suggesting that this is an example of divergent evolution (Kawai 2009). However, some believe that the conservation is coincidental between the Z chromosomes of birds and *G. hokouensis* since other lizards, such as the Australian dragon lizard, show no homology to other ZW chromosomes and seem to be an example of convergent evolution (Ezaz *et al*. 2009a; Ezaz *et al*. 2009b). Due to the homology of ZW chromosomes of the chicken (with a *Dmrt1* sex-determining gene) and *G. hokouensis*, it seems likely that *Dmrt1* plays an upstream role and serves a similar purpose in this gecko species (Smith *et al*. 2009). *G. japonicus* also has the *Dmrt1* gene present on the 6th chromosome (Trifonov *et al*. 2011). However, unlike *G. hokouensis*, *G. japonicus* is thought to have a TSD mechanism (Tokunaga 1985, Ding *et al*. 2012).

There are 3 different TSD patterns. Some reptiles display a female sex bias at low temperatures and a male bias at high temperatures. This is known as a FM pattern. When there is a female bias at high temperatures and a male bias at low temperatures, it is known as an MF pattern. The last pattern is known as FMF, which is when higher and lower temperatures have a female bias and intermediate temperatures display a male bias. This is the proposed TSD mechanism for *G. japonicus* (Tokunaga 1985; Ewert *et al*. 1994). However, there has been much speculation as to whether *G. japonicus* is truly a TSD species. Past research shows conflicting evidence for the presence and absence of sex chromosomes in this species (Nakamura 1932; Yoshida & Itoh 1974; Chen *et al*. 1986). There are many speculations as to why these differences were observed, such as a hybridization of the species, the species may have been incorrectly identified, or separation of species (Japanese versus Chinese *G. japonicus*) has led to a cryptic species (Sarre *et al*. 2004; Ezaz *et al*. 2009; Gamble 2010). Also, because reptiles with GSD mechanisms can be overridden by temperature, it makes it harder for us to really know whether *G. japonicus* is truly TSD, or is GSD with a TSD override at extreme temperatures.

In a more recent study it was found that there were significantly more females at the high and low end of the temperature spectrum. However, the intermediate temperature displayed almost a 1:1 ratio, which is not consistent with the TSD mechanism proposed earlier by Tokunaga in 1985. When observing egg size they found that larger eggs are more likely to produce females and when looking at the intermediate temperature the larger eggs also produced males. These observations tend to support the old FMF TSD mechanism (Ding *et al*. 2012).

For my study I would like to see how some sex-determining genes are involved in this TSD species. We now know how yolk hormones, egg size and temperature affect the sex of *G. japonicus* offspring, but little information has been garnered about the gene expression. As such, my goal is to find how these genes may differ in expression from the different temperature regimes and further support the TSD mechanism of *G. japonicus.* If there is a female bias at extreme temperatures (high and low), then I expect significantly more samples to show low *Dmrt1* transcription activity. Whereas at the intermediate temperature, if there is a male bias, then I expect more samples to show an increase in transcription activity in the *Dmrt1* gene.

Materials and Methods

Egg collection

Gravid female geckos were caught around Nanjing, Jiangsu Province, China between late May and early August 2013. Female geckos were housed in a 200 x 150 x 150 mm plastic mesh cage in the Nanjing Normal University Herpetology Laboratory in East China. Housing temperature was maintained between 25-30°C and their diet consisted of Small mealworms (*Tenebrio molitor*). Paper was set down in the cages so eggs will stick to it for easy removal (Ding *et al*., 2012). After removal, which occurred twice daily, eggs were weighed and measured. In clutches that contained two eggs, which were usually stuck together, the eggs were weighed simultaneously and the mass was divided in half. Eggs were then randomly selected to be incubated at either 24°C, 28°C or 32°C (±0.2°C). These temperatures allow for lowest mortality rates while still generating significant data (Tokunaga 1985; Kratochivíl *et al*. 2008; Ding *et al*. 2012). Incubated eggs were placed in a plastic box containing dry vermiculite and each incubator

contained a cup of water for humidity purposes. Females were then weighed again, measured and released at their original locations. Capture of females as well as incubation of eggs and tissue samples were approved by the Central Washington University Institutional Animal Care and Use Committee (IACUC protocol # A051303) and complied with the Chinese regulations through permits granted to the Herpetology Laboratory at Nanjing Normal University.

RNA collection

When the embryos reached their specific interval of incubation (either 1/3 development or 2/3 development), eggs were cracked and the embryos were teased apart from the yolk and then placed into sterile centrifuge tubes. Then 40µl of RNA*later* was placed in the centrifuge tube to preserve the RNA in the embryo's tissue. The total embryonic RNA was extracted using the reagent RNAiso Plus (TaKaRa) according to manufacturer's specifications. Then the extracted RNA was stored at -80°C. Purity of RNA samples was assessed using the Nanodrop 2000c and running the samples on a 1% agarose gel containing ethidium bromide.

To convert RNA into cDNA a PrimeScript RT reagent Kit (TaKaRa) was used with RNA at a concentration of 500ng/10µl in a 10µl reaction system, then following manufacturer specifications, the following polymerase chain reaction (PCR) settings were used to complete conversion: 37°C for 15 min (reverse transcription), then 85°C for 5 sec (inactivation of reverse transcriptase with heat treatment) for one cycle. cDNA was stored it at -20°C until further use.

Designing Primers

I started off by doing a BLAST search in the National Center for Biotechnology Information (NCBI) database for *Dmrt1* and took similar reptile sequences into the software program Sequencher. From there I found primers that had similar melting points and would lead to less than 200bp products. The following primer pair seemed to work best: forward primer 5'-CCGTCATGGTATGAGTTCCC-3', reverse primer 5'-GGCTTTTGTTTCAGAGAAGG-3' leading to a 142bp product.

Quantitative PCR

For quantitative reverse-transcriptase polymerase chain reaction (Q-PCR) I used the Bio-Rad iQ5 system cycler to run my samples. Q-PCR reactions were performed in a final volume of 20µl with the following mix: 10µl SsoAdvanced Universal SYBR Green Supermix (BioRad), 0.5 µl of 20mM forward primer, 0.5 µl of 20mM reverse primer, 1µl cDNA template, 8µl deionized water. The Q-PCR settings included an initial denaturation cycle at 95°C for 30 seconds, followed by 40 cycles at 95°C for 5 seconds and 60°C for 15 seconds, and a final elongation cycle at 72°C for a minute. Expression levels of *Dmrt1* were normalized to the housekeeping gene *Ef-1α* using forward primer 5'-CCTTCAAATATGCCTGGGT-3' and reverse primer

5'-CAGCACAGTCAGCTTGAGAG-3' as used by Wang et. al, in 2011. Negative controls were also run during each reaction that excluded the cDNA template.

Sequencing

cDNA was sent out to Operon (Louisville, KY) with aforementioned *Dmrt1* and *Ef-1α* primers to be sequenced. The sequences were then compared in a BLAST alignment search using the NCBI database.

Statistical Analysis

Chi-square analysis in the statistics program R version 2.15.1 was used to determine if there was a significant difference between the proportions of males to females per temperature regime. Welch's two-sample t-test was used to compare the egg mass to embryo survival rate. Mann-Whitney U test had to be used to compare the mass of males and female hatchlings due to abnormal data at 28°C. When comparing multiple mean days to hatching between studies, a general linear model was used in RStudio version 0.98.113. Then SigmaPlot was used to graph smaller than average versus larger than average egg sizes. To compare the body mass of females to the eggs they laid, a Spearman's correlation analysis was conducted. The alpha level for all statistics was set at 0.05 a priori, and normality was tested for each test.

Results

Hatching Success

The study began with 412 eggs laid in the lab and ended the study with only 152 samples of cDNA and 57 living geckos leading to an overall survival rate of 50.7%. 352 of those 412 eggs survived being laid and began incubation, meaning there was a 15% initial loss. When comparing the egg mass of embryos that died with the embryos that

lived, there was a significant difference between them, with the mean mass of deceased embryos less than the mean of surviving embryos (t = 8.3991, df = 359, P = 1.06e-15).

Table 1 Displays the sample size of each development group per temperature regime, and the survival for each of these groups.

Development Stage	Temperature (°C)	Sample Size	Survival (%)
1/3rd	24	31	97
1/3rd	28	22	67
1/3rd	32	14	44
2/3rd	24	31	86
2/3rd	28	22	63
2/3rd	32	26	68
hatching	24	26	63
hatching	28	14	45
hatching	32	21	60

At 24°C, 63 % (26/41) of the embryos survived until hatching and up to 120 days later when they were sexed. At 28°C, 45% (14/31) of embryos survived hatching, and at 32°C, 60% (21/35) of embryos survived hatching and sexing (Table 1). Out of the 16 surviving double clutch hatchlings, 11 were both female (68.75%) and one was strictly male (6.25%, at 32°C). The other four double clutches were siblings of the opposite sex (25%, with 50% of those at 28°C). There was no significant difference between the total egg mass of male or female hatchlings for all temperature regimes (U = 289, N = 61, P = 0.9349), nor was there a difference comparing sexes at 24°C (U = 43, N = 26, P = 0.5147), at 28°C (U = 16, N = 14, P = 0.6191) or 32°C (U = 39.5, N = 21, P = 0.2269). To compare the ratios to other studies, mean hatching days were observed. A general linear model shows that there is no significance in the difference between this research

and the research conducted by Ji *et al*. 1991, and Ding *et al*. 2012 ($t = 0.262$, $df = 8$, $P = 0.8023$), although there is a difference in the days it takes to hatch between temperature regimes ($t = 8.185$, $df = 8$, $P = 0.000179$).

Hatchling Sex Ratio

At 24°C, the proportion of females was higher than males (80.77%, $\chi^2 = 9.85$, $df = 1$, $P = 0.0017$). Similarly, at 32°C a greater proportion of females hatched (85.71%, $\chi^2 = 10.71$, $df = 1$, $P = 0.00106$). However, at 28°C there was not a significant difference between the proportion of female versus male hatchlings (71.43% females to 28.6% males, $\chi^2 = 2.57$, $df = 1$, $P = 0.1088$, Figure 2). When comparing below average and above average egg size, we find that all ratios were more than 50% female (Figure 3) and no difference was found between proportion of females from above average versus below average egg sizes for all temperature regimes (At 24°C, $\chi^2 = 1.36$, $df = 2$, $P > 0.05$; At 28°C, $\chi^2 = 0$, $P > 0.05$; At 32°C, $\chi^2 = 1.63$, $P > 0.05$). However, when observing data at 32°C we get a p-value of 0.01246 when using a Welch's two-sample t test. So I plotted my means and found that the standard deviation error bars do overlap, meaning that this data may or may not be significant (Whitlock & Schluter 2009). Also there was a significant correlation between the body mass of the mother and the initially laid egg mass of the offspring ($n = 393$, $r = 0.41358$, $P < 2.2e\text{-}16$).

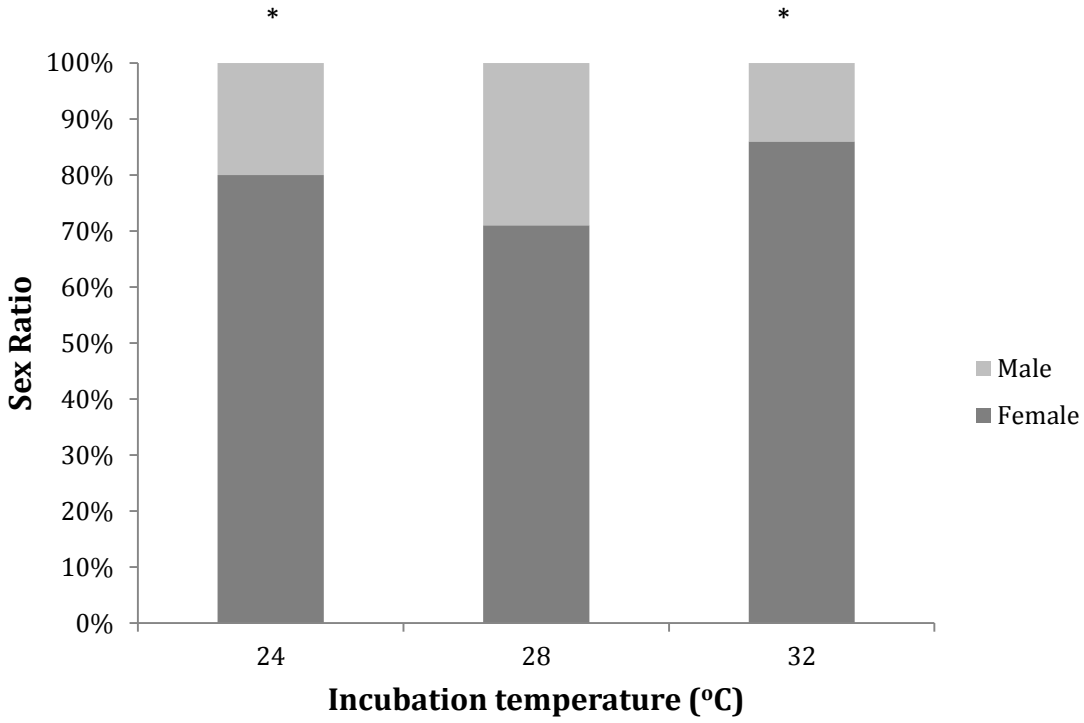

Fig. 1 Effect of temperature on sex ratios. The effect of temperature on sex ratios in *G. japonicus* shows a clear female hatchling bias at 24°C and 32°C. *$P < 0.05$

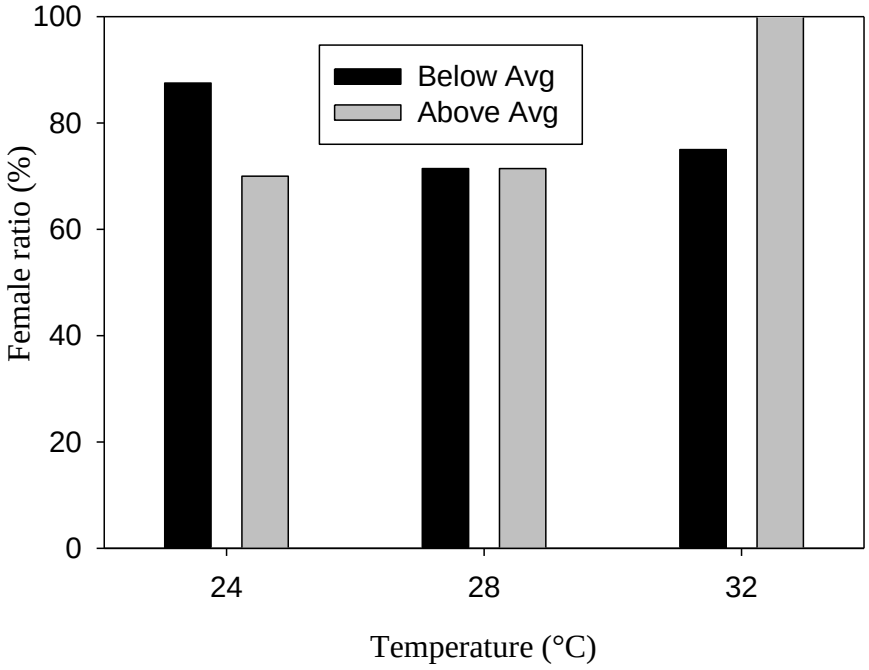

Fig. 2 Female sex ratio compared to egg mass. Displays female sex ratios in regard to egg size, and whether the egg was above or below the mean mass (g) for each temperature regime.

Sequencing

Dmrt1 sequence was aligned and matched with *Gekko hokouensis*. The sequence of *G. japonicus* was 97% identical to that of *G. hokouensis* with only three mismatches out of 91 base pairs (Figure 4).

Score	Expect	Identities	Gaps	Strand
152 bits(82)	6e-34	88/91(97%)	0/91(0%)	Plus/Plus

```
Query  1    CTCCTACTTGGGACAGAGTGTGGGCACCCCTGCCTGCGTTCCCCAGATTTTGACTTTTGA  60
            ||||||||||||  ||||||||  ||||||||||||||  ||||||||||||||||||||
Sbjct  538  CTCCTACTTGGGCCAGAGTGTTGGCACCCCTGCCTGTGTTCCCCAGATTTTGACTTTTGA  597

Query  61   GGAGAGTCCTTCCTTCTCTGAAACAAAAGCC  91
            |||||||||||||||||||||||||||||||
Sbjct  598  GGAGAGTCCTTCCTTCTCTGAAACAAAAGCC  628
```

Fig. 3 Alignment of DMRT1 sequences. Displays the alignment of *Dmrt1* sequences from *G. japonicus* (Query) and *G. hokouensis* (Sbjct).

Q-PCR products

There were no obtainable Q-PCR results from the Bio-Rad iQ5 software. However when a gel of the products was run it was found that the sample NJ13 displayed faint bands at 142bp and bright bands at 180bp (Figure 5).

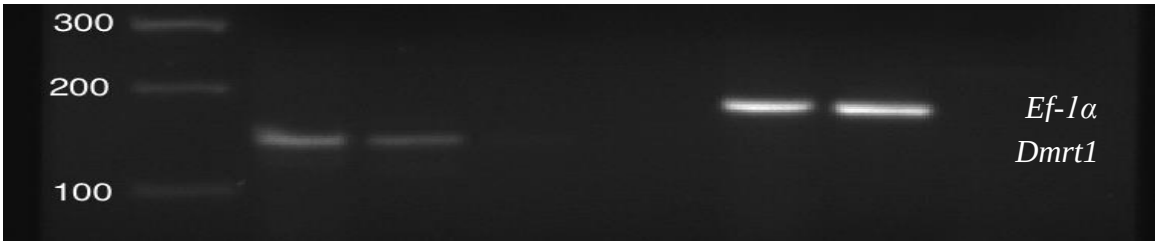

Fig. 4 PCR analysis of genes. Electrophoresis 2% agarose gel run at 120V for 45min. This figure depicts the product of the *Dmrt1* gene (142bp) and *Ef-1alpha* gene (180bp) for sample NJ13 which was incubated at 32°C and incubated at 2/3rds development. The numbers on the left hand margin display the band lengths from the DNA ladder.

Discussion

Approximately 200 MYA ago, lizards and birds shared a common ancestor (Graves & Shetty 2001). Interestingly, the ZW sex chromosomes of *G. hokouensis* are highly conserved to those of avian species. This includes the *Dmrt1* gene (Kawai *et al.* 2009). Also in 2009, Smith and colleagues found that the *Dmrt1* gene is important to the sex determination of chickens (*Gallus gallus domesticus*), and some even consider *Dmrt1*

as a key sex-determining gene for chickens (Smith *et al*. 2009). It seems plausible that *Dmrt1* could contribute to the sex determination of *G. hokouensis,* and possibly other closely related *Gekkonidae* species with other sex-determining mechanisms.

Additionally, the results from research conducted on *G. japonicus* are highly conflicting and do not give much light into this species' sex-determining mechanism (Gamble 2010). As such, I hypothesized that the conservation of the *Dmrt1* gene extended to that of the TSD sex-determining mechanism of *G. japonicus,* instead of being limited to GSD individuals. Sure enough, in Figure 4 the two *Dmrt1* sequences of *G. hokouensis* and *G. japonicus* are 97% identical. This shows that the sequence of the *Dmrt1* gene is highly conserved between these species even though they may have different sex-determining mechanisms.

The difference between GSD and TSD individuals may not be that different at all, especially when comparing a ZW system to a TSD mechanism. It seems plausible that these two systems use a similar gene dosage mechanism, because it seems that gene dosage systems are easier to override than dominant systems (Quinn *et al*. 2007;Ezaz *et al*. 2009b). If this is the case then it would not seem so farfetched to think that TSD systems use similar genes, such as *Dmrt1,* like GSD systems, with temperature influencing the dosage of the sex-determining genes.

Additionally, this study reassessed the TSD sex bias displayed at various temperature regimes for *G. japonicus*. However, there were many stages of potential egg loss throughout the experiment and only 50.7% of all eggs laid survived to reach their specified groups' development stage. Now, when looking at hatching groups alone, 28°C

had the lowest mortality at only 48% in survival rate. Whereas 24°C and 32°C had survival rates just above 70%. When looking at Ding et al.'s research conducted in 2012, I expected a survival rate of about 80 percent per temperature regime. I did find that eggs with a lower mass tend to decease more often than eggs with a higher mass. Some factors that may have contributed to my loss could include some eggs simply being infertile when placed in the incubators (Tokunaga 1985). Also, eggs were lost during the measuring process by accidently getting cracked. What contributed largely to the egg loss observed was a mold infestation during incubation. To combat the mold I cleaned all materials used with ethanol. Also, instead of keeping humidity of approximately -220kPa I used dried vermiculite and included a container of water present in the incubator at all times with my samples, which decreased the amount of eggs that molded.

Egg mass was not significantly different for males versus females at any temperature regime when using a Mann-Whitney U test, which only was used since the data at 28°C could not be normalized. However, when observing data at 32°C using a Welch's two-sample t test we get a significant p value. Therefore, the mass of female-producing eggs tends to be larger than the mass of male-producing eggs at 32°C. This is also visually shown in Figure 3. Unfortunately, the data could not be normalized at 28°C so a non-parametric test had to be used. When comparing the proportions of males to females for above average and below average eggs there was no difference. Also, when observing egg mass we find that the initially laid egg's mass had a significant correlation to the mother's mass. Therefore, larger mothers tended to produce larger eggs. However, a non-parametric test had to be used instead of the more powerful Pearson correlation, because the data could not be transformed to show normality due to some outliers.

Consistent with Tokunaga's research in 1985 (93% females at 24°C and 76% females at 32°C) and Ding *et al*. 2012 (88% females at 24°C and 85% females at 32°C), at 24°C I had a female bias of 81% and at 32°C a female bias of 86%. Similar to Ding's research I did not see a male bias at 28°C, unlike Tokunaga's research. However, unlike Ding's research in 2012, I did not see a significant difference between female sex ratios above or below mean egg size. Instead, I found that at 32°C all male hatchlings came from below average egg sizes (Figure 3), which would explain the large difference we see between average female and average male egg sizes in Figure 1.

This suggests that the species is then a F-M bias with the female bias at low temperature and a male bias at higher temperatures. The reality is that this species is much more cryptic than once assumed. When we take egg size into account it can alter our perception on the sex bias. It seems that the egg size may have a direct effect on the sex of an embryo in some species (Müller *et al*. 2005; Radder *et al*. 2009; Ding *et al*. 2012). Even though only males hatched at 32°C in smaller eggs, smaller eggs still had a higher ratio of females. Also at 28°C there was not a higher proportion of males hatched in smaller eggs rather than larger than average eggs (Fig.3). To me this seems that at the average temperature at which the eggs hatch there is an expected 1:1 male female bias, which would be consistent with a GSD mechanism. However, at extreme temperatures I find that there were significantly more females than males (Fig. 2). My findings suggest that this species may have a GSD sex-determining system and simply displays a TSD override as observed in other species (Shine *et al.* 2002; Quinn *et al*. 2007; Radder *et al*. 2008). However, more research should be done on the sex bias and egg size to support this sex bias pattern.

There are many differences observed in this species, whether its sex determining system is GSD or TSD (Yoshida & Itoh 1974; Tokunaga 1985; Chen *et al*. 1986; Ding *et al*. 2012), or the type of TSD displays (Tokunaga 1985, Ding *et al*. 2012). Could it then be possible that the species is simply evolving into two independent species, or have there simply been mistakes in collecting and identifying the correct species (Gamble 2010)? Without this information, and the information of where Yoshida and Itoh collected their samples, it will remain a mystery of whether these geckos indeed have an XY sex-determining system. However, with new technology it may be easier than ever to answer these questions. Recently RAD-seq has been used to identify geckos that may be using GSD or TSD systems. This technology makes detecting sex chromosomes easier than the previously used karyotyping method. Not only has it led to better understanding in which geckos have sex chromosomes present and which do not, but it also has identified that in geckos there is a large amount of transitions between sex-determining systems (Gamble *et al*. 2015). This article also supports my idea of *G. japonicus* having a GSD system.

Unfortunately, the qPCR portion of my research has not been completed due to technological difficulties. There was a substantial amount of trouble shooting, such as re-calibrating the iQ5 cycler and changing the temperatures and lengths of time each step took. I also tried shortening the product bp lengths by designing new primers with the known sequence, but that also made no difference. In the end I decided to run the qPCR product from sample NJ13 on an electrophoresis gel. With a 2% agarose gel, enough separation occurred that we could see distinct bands at approximately 142 bp. This is the expected length of our *Dmrt1* product, which shows that the gene is present and is being

expressed. Although without the qPCR results I will not know how much relative to other samples and other housekeeping genes that are expressed the same, no matter the sex of the organism.

In the end it would seem that there is a substantial overlap between sex determining mechanisms. What used to be thought of as simply GSD versus TSD and dominant genes versus dosage effects is not so easily distinguished. Even if *SRY* is the initial sex-determining factor in mammals, and *Dmrt1* in chickens, there are many other genes and events that have to take place to complete the development of testis (Larney *et al*. 2014). There are many factors that could disrupt this development, such as environmental influences, steroids and gene mutations (Bull 1980; Rhen & Lang 1994; Kent *et al*. 1996). What is to say that temperature is not just simply regulating the transcription of the *Dmrt1* gene, which then triggers other genes such as *Sox9* to promote the development of testis.

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